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# The relevance of covalent binding to mouse liver DNA to the carcinogenic action of hexachlorocyclohexane isomers

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## Abstract

**[$^3$H]Hexachlorocyclohexane (HCH) was synthesized by chlorination of [$^3$H]benzene prepared by catalytic tritiation of benzene with tritiated water. The isomers of HCH were separated by adsorption chromatography on silica gel. In order to determine the covalent binding to DNA, [$^3$H]HCH was administered to male mice by oral gavage, and liver DNA was isolated via chromatin. The specific radioactivity of the DNA was normalized by the dose administered and expressed in the molar units of the Covalent binding index, CBI = DNA damage/dose = (μmol bound HCH/mol DNA nucleotide)/(mmol HCH administered/kg body weight). CBI values of ~0.2 were found 10 h after the administration of alpha- and gamma-HCH. Enzymatic digestion of the DNA to the nucleosides and h.p.l.c. analysis revealed that ~40% of the radioactivity co-migrated with the natural nucleosides. At elution volumes known to contain the more lipophilic carcinogen-nucleoside adducts, ~10% of the radioactivity could be detected. The remaining 50% of the radioactivity eluted with the front, representing a mixture of oligonucleotide-HCH adducts and/or hydrophilic degradation products which were strongly but not covalently associated with intact DNA. Therefore, a true CBI of 0.02–0.1 must be expected both for alpha- and gamma-HCH. This CBI is by a factor of $10^5$–$10^6$ below the value found with the strongest DNA-binding carcinogens like aflatoxin $B_1$ or dimethylnitrosamine and is unlikely to be decisive for the liver tumor induction in mice because of the following additional findings: (i) Both isomers gave rise to similar levels of DNA damage although the alpha-isomer is a much more potent tumor inducer. This similarity was seen not only at the time of maximum binding but up to 10 days after oral administration; (ii) three mouse strains with apparently different susceptibility to tumor induction by gamma-HCH could not be distinguished with respect to DNA binding; (iii) the level of DNA binding of alpha-HCH (CBI = 0.02–0.1) is more than three orders of magnitude lower than would be expected if the mechanism of tumor induction was by genotoxicity mediated by DNA-binding. For a preliminary investigation on a potential stimulatory effect on liver DNA replication and cell division, [$^{14}$C]thymidine was administered i.p. 3.5 h before sacrifice of the [$^3$H]HCH-treated mice. The alpha-isomer was found to be more potent than the gamma-isomer in this respect. Taken together, our data allow the conclusion that the non-mutational processes must be more important for the carcinogenicity of HCH.**

## Introduction

Hexachlorocyclohexane (HCH)* comprises of a group of isomers of which the gamma-isomer, later called lindane, has very useful pesticidal activity (1). HCH have become of great public concern because the lindane batches used in the late forties contained appreciable concentrations of alpha- and beta-isomer. The alpha-isomer was found to induce liver tumors in rats and mice (2), the beta-isomer was found to have very low biodegradability and to be deposited in animal fat. Although the lindane batches used since the fifties were at least 99% pure gamma-isomer, a new discussion arose from controversial findings of a liver tumor-inducing potential of lindane itself.

Chemically-induced tumors are now thought to be the result of a DNA damage succeeded by appropriate promotion (3). Most chemicals exert their activity by covalent interaction of a reactive metabolite with DNA in the target organ and are therefore called genotoxic. The metabolism of HCH involves the formation of olefins (1) and a subsequent epoxidation could result in the generation of an electrophilic species.

Another group of tumor-enhancing agents, viz co-carcinogens and promoters, do not themselves react with DNA but apparently modulate one or several out of a variety of biochemical and biological steps related to the process of tumor formation. Such activities are also discussed for HCH. For instance, alpha-HCH was found to enhance the proliferation of putative preneoplastic cells in rat liver (4), and all HCH isomers are known to be inducers of drug-metabolizing enzymes (1), the alpha-isomer being more potent than lindane.

It was the aim of this study to provide more information about the mechanism of tumor induction by HCH. For this reason it was examined whether the isomers of HCH can be metabolized *in vivo* to reactive metabolites able to reach and bind to liver DNA or whether the hepatocarcinogenicity is rather due to non-genotoxic effects. It seemed especially worthwhile to investigate whether the clear difference between the alpha- and the gamma-isomer with respect to biological effects and tumor induction (2) was reflected in their ability to bind to DNA, and whether the apparent difference in susceptibility of different strains of mice to the carcinogenicity of gamma-HCH (5–7) can be based upon different levels of DNA binding.

## Materials and Methods

### *Chemicals and apparatus*

Reagents without specified distributor were of the highest purity available from Merck, Darmstadt, FRG. Hydroxylapatite (HA) (DNA-Grade, Bio-Gel HTP) was purchased from Bio-Rad, Richmond, CA, sodium dodecyl sulfate (SDS) from Sigma, St. Louis, MO, Nonidet P 40 (NP 40) and copper oxide (wire form) from BDH Chemicals Ltd., Poole, UK. Carrierfree [$^3$H]$_2$ and [$^{14}$C]thymidine with a sp. act. of 61 mCi/mmol were purchased from the Radiochemical Centre, Amersham, UK. Desoxyribonuclease I (E.C. 3.1.4.5.) from bovine pancreas, phosphodiesterase I (E.C. 3.1.4.1.) from *Crotalus atrox* venom and alkaline phosphatase III (E.C. 3.1.3.1.) from *Escherichia*

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*Abbreviations: HCH, hexachlorocyclohexane; CBI, Covalent binding index; PBI, Protein binding index; II, Incorporation index; CIP, chloroform/isoamyl alcohol/phenol (24 + 1 + 25 vol.); SDS, sodium dodecyl sulfate; HA, hydroxylapatite.

*coli* were obtained from Sigma, St. Louis, MO. Dialysis tubing (Visking type 20/32, mol. wt. exclusion at 12 000–14 000 daltons; diameter 17 mm) was from Union Carbide, Chicago, IL. The u.v. lamp was a Mineralight UVSL-58 50W from Ultra-Violet Products Inc., San Gabriel, CA.

Radioactivity measurements were carried out in 10 ml Insta-Gel (Packard Instruments, Downers Grove, IL) in a liquid scintillation counter, Packard Tri Carb 460 CD equipped with and calibrated for the automatic analysis of [$^3$H]/[$^{14}$C] double-labelled samples.

The isomeric and radioactive purities of the isomers of HCH were determined on a semipreparative gas chromatograph, Carlo Erba, Fractovap Linea 2200 (Carlo Erba, Rodana, Milano, Italy). The mass spectrum of HCH was recorded at the Institute of Organic Chemistry, ETH Zürich, Switzerland.

The h.p.l.c. analysis of the nucleosides were performed on a semipreparative column (250 mm x 8 mm ID) equipped with two h.p.l.c. pumps, Kontron, LC Pump 410 (Kontron, Zürich, Switzerland), controlled by a Kontron Programmer 200, for generating a linear gradient of two eluants.

*Synthesis of [$^3$H]HCH*

[$^3$H]Benzene was prepared by catalytic exchange tritiation of benzene on a high-vacuum line. [$^3$H]$_2$O derived from combusting 8.8 Ci carrier-free [$^3$H]$_2$ at 600°C over 30 g copper oxide (wire form) was trapped under high vacuum in 250 µl trifluoroacetic acid cooled with liquid nitrogen. This mixture was lyophilized into a break-seal ampoule containing 500 µl (430 mg, 5.5 mmol) benzene and 10 mg Platinum Black. The sealed ampoule was incubated for 6 days at 100°C.

[$^3$H]HCH was synthesized by chlorination of [$^3$H]benzene (8). 16.5 mmol chlorine gas were generated from oxidation of 33 mmol silver chloride with 12 g potassium dichromate in 100 ml concentrated sulfuric acid and trapped by cooling with liquid nitrogen. The [$^3$H]benzene and chlorine were lyophilized into a 25 ml quartz round-bottom flask prefilled with 5 ml carbon tetrachloride. The mixture was kept at −25°C to −30°C for 2 h under irradiation at 254 nm. Solvent and unreacted benzene were distilled off and the residue containing ~50% of the radioactivity was extracted with petroleum ether (30–45°C boiling point) to bring the alpha-, gamma-, delta- and epsilon isomers into solution. The specific radioactivity of HCH was calculated from results derived in preliminary synthesis with a trace amount of radioactivity and was found to be ~1 Ci/mmol. The products were identified as HCH by mass spectrometry. The isomers were separated by chromatography on silica gel 60 according to Granger and Zwilling (9). The radiochemical purity of the isolated isomers was >99%, the isomeric purities were checked by semipreparative gas chromatography. Column: OV 17, 2% methylphenylsilicone, 200 cm; column temperature: 175°C; injection temperature: 210°C, $N_2$-pressure: 0.9 kg/cm$^2$) and was found to be 98% (alpha-HCH). 92% (gamma-HCH; 8% epsilon-HCH) and 95% (delta-HCH; 5% epsilson-HCH). Beta-HCH was prepared by recrystallisation from a chloroform extract of the residue. The radiochemical purity was >99%, the isomeric purity was 96% (4% epsilon-HCH).

*Animals and treatments*

Young adult male mice of the strains NMRI, CF1, and C6B3F1 with weights ranging from 25 g to 40 g were obtained from Celamerck, Ingelheim, FRG. Laboratory chow (No. 21-343-7, Klingenthal Mühle AG, Basel, Switzerland) and tap water were provided *ad libitum* for one week for acclimatisation. Administrations of HCH were carried out between 9.00 and 10.00 a.m. by oral gavage in polyethylene glycol 300 containing 5% ethanol. The animals received an i.p. injection of 5–15 µCi [$^{14}$C]thymidine in 0.9% NaCl 6.5 h later and were killed after another 3.5 h. For the determination of the time dependence of the DNA binding, the animals did not receive [$^{14}$C]thymidine and were sacrificed 1, 3, 5 and 10 days after the [$^3$H]HCH administration.

*Isolation of DNA and chromatin protein*

*Isolation of chromatin.* (In the cold) Animals were bled by open heart puncture under ether anaesthesia. The livers were exised, minced and homogenized in a teflon Potter-Elvehjem-type homogenizer in 3–4 volumes 75 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl pH 7.8. Crude chromatin was prepared according to Yaneva and Dessev (10) with some modifications. After adding a solution of 2% (v/v) of the non-ionic detergent Nonidet P 40 up to a final concentration of 0.2% (v/v) the samples were incubated at 4°C for 15 min. Chromatin was precipitated at 700 *g* for 5 min. The pellet was washed once with 75 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl pH 7.8 and centrifuged for 5 min at 3500 *g*. This pellet contained about 2–3 mg DNA and 20–30 mg protein/g liver.

*Isolation of DNA.* (Room temperature) DNA was isolated from chromatin according to Markov and Ivanov (11) with some modifications. The chromatin pellet was suspended in 25 ml lysing medium (8 M urea, 0.24 M sodium phosphate buffer pH 6.8, 10 mM EDTA, 1% (w/v) SDS) and homogenized on a Waring blender in a custom-made air-tight aluminium vessel (46 mm diameter, 28 mm high) run at high speed for 30 s and then cooled in cold ethanol (−40°C) for 15 s. The blending-cooling procedure was repeated 4 times. After cautious transfer into a 50 ml polypropylene centrifuge tube the foam was broken by centrifugation for 3 min at 1000 *g*. 10 ml CIP (480 ml chloroform, 20 ml isoamyl alcohol, filled up to 1 liter under stirring with warmed liquid phenol) was added and proteins were extracted in the centrifuge tube under extensive shaking for 10 min on a shaking machine. The resulting suspension was separated into two layers by centrifugation for 15 min at 20 000 *g*. The CIP phase was saved for protein isolation, the supernatant aqueous layer was first decanted and then pipetted into a new polypropylene centrifuge tube and was extracted once more with 10 ml CIP. The aqueous solution was extracted twice with 25 ml ether in a 250 ml separating funnel for the removal of trace amounts of phenol, was left standing overnight at room temperature and was applied to an HA column. Dry HA (1 g/g liver) of special batches tested for high absorptivity of DNA was suspended overnight in filtered MUP (8 M urea, 0.24 M sodium phosphate buffer pH 6.8), the slurry was swirled gently and was left untouched for 10 min. The fine particles were decanted. The remaining slurry was poured into 25 x 120 mm glass columns and the MUP was let run off. The aqueous nucleic acid solution was loaded on the column and the elution was monitored at 260 nm. Proteins were washed from the column with filtered MUP at a flow rate of 1–2 ml/min by gravity until the transmission had returned to background value. To avoid a mixing of the eluants the column was let run dry before purging the urea from the column with two bed volumes 14 mM sodium phosphate buffer, pH 6.8. DNA was eluted with 0.48 M sodium phosphate buffer, pH 6.8, and ~20 ml of the DNA solution were collected. From here on, extreme caution is required not to use glassware, equipment or facilities which are also used for procedures involving high radioactivity levels. The sample was dialyzed at 4°C against 10 liter 0.2 M NaCl overnight. DNA was precipitated by adding 2 volumes ethanol and keeping at −20°C for at least 12 h. The DNA was centrifuged for 20 min at 1000 *g*, the supernatant was decanted and DNA was dried *in vacuo* for 2–3 h. The highly purified DNA was dissolved in 10 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.0. The amount of DNA was determined on the basis of an absorbance of 20 at 260 nm for a solution of 1 mg DNA/ml. The yield of DNA was ~1 mg/g liver limited by the use of suboptimal amounts of hydroxylapatite. The contamination of the DNA by protein was <0.2% as derived from liver-DNA isolations from animals treated with L-[$^{35}$S]methionine or with L-[$^3$H]lysine.

In a control experiment, DNA was isolated from the pooled livers of two mice treated with [$^3$H]HCH and [$^{14}$C]thymidine. After each purification step DNA was precipitated from an aliquot of the aqueous solution by adding 2 volumes ethanol at −20°C. After a centrifugation for 20 min at 1000 *g*, DNA was dried *in vacuo* and dissolved in deionized water. The amount of protein contaminating the crude DNA was measured by the method of Bensadoun and Weinstein (12), the amount of DNA was calculated from the specific [$^{14}$C]activity. The specific [$^3$H]activity of the DNA was calculated as (total [$^3$H]activity − [$^3$H]activity of protein)/amount of DNA.

*Isolation of chromatin protein.* 1 ml of the first CIP extract from a DNA isolation of 10 g liver was shaken with ~5 ml 1% SDS in 14 mM sodium phosphate buffer, pH 6.8. Protein was precipitated with 25 ml acetone and washed 5 times by redissolving in 2 ml 1% SDS and acetone precipitation. The final protein sample in 1% SDS was diluted with water to 0.1% SDS, was precipitated by the addition of 2 volumes acetone and was stored at −20°C overnight. After a centrifugation at 300 *g*, the supernatant was decanted and the protein residue was freed from acetone *in vacuo* for ~15 min. Protein was dissolved in 2 ml 1% SDS in 14 mM sodium phosphate buffer, pH 6.8, overnight and the solution was diluted with water to a final concentration of 1.4 mM sodium phosphate buffer. The amount of protein was determined with the Folin reagent. 1–4 ml containing ~0.5 mg protein/ml were used for the liquid scintillation counting.

*Isolation of HCH metabolites*

The supernatant of the first acetone precipitation of chromatin protein from the CIP phase was dried *in vacuo*. About 75% of the radioactivity in the CIP phase could be dissolved in 10 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.0 and was loaded on a Lichrosorb $RP_{18}$ column also used for the analysis of nucleosides by h.p.l.c. (see below).

Water-soluble metabolites were obtained from the aqueous solution after the first CIP extraction of chromatin homogenate. DNA was precipitaed by the addition of 2 volumes ethanol and the supernatant was dried *in vacuo*. After dissolving the residue in 10 mM $MgCl_2$ 10 mM Tris/HCl, pH 7.0 the sample was analysed by h.p.l.c.

*H.p.l.c. analysis of the nucleosides*

DNA (1–2 mg/ml) in 10 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.0 was digested enzymatically by the method described by King *et al.* (13). The resulting deoxynucleosides were separated by h.p.l.c. on a Lichrosorb $RP_{18}$

column (250 mm x 8 mm) with a distilled water/methanol gradient of 0–10% methanol in 5 min, 10% methanol for 5 min and 10–100% methanol in 45 min at a flow rate of 3.5 ml/min. The optical density of the eluate was recorded at 254 nm. Fractions of 2 min were collected and the scintillation counting was performed after the addition of 10 ml Insta-Gel. To avoid phase separation between the scintillation cocktail and the eluate, the fractions 11 – 14 were diluted with 1 ml methanol. The retention times of the natural deoxynucleosides deoxycytosine, deoxyguanosine, thymidine and deoxyadenosine were 9 min, 11.5 min, 13 min and 18 min, respectively. Recovery of radioactivity eluted, as a fraction of the injected ranged between 90 and 110%, both for [$^3$H] and [$^{14}$C].

*Calculations and statistics*

*Determination of CBI.* The radioactivity in the DNA after treatment of mice with [$^3$H]HCH was expressed after normalization to the dose administered:

$$CBI' = \frac{\text{d.p.m./mg DNA}}{\text{d.p.m./kg body weight}}$$

This value was converted to the molar units,

$$CBI = \frac{\mu\text{mol chemical bound/mol DNA nucleotide}}{\text{mmol chemical applied/kg body weight}}$$

according to CBI = CBI' x 309 x $10^6$ on the basis of an average mol. wt. of 309 g/mol DNA nucleotides (14).

*Determination of Protein binding indices, PBI.* The radioactivity in the chromatin protein after treatment of mice with [$^3$H]HCH was expressed after normalization to the dose administered:

$$PBI' = \frac{\text{d.p.m./mg chromatin protein}}{\text{d.p.m./kg body weight}}$$

This value was converted to the molar units,

$$PBI = \frac{\mu\text{mol chemical bound/mol amino acid}}{\text{mmol chemical applied/kg body weight}}$$

according to PBI = PBI' x 110 x $10^6$ on the basis of a mol. wt. of 110 g/mol for an average amino acid (15).

*Determination of Incorporation indices, II.* The radioactivity in the DNA after treatment of mice with [$^{14}$C]thymidine was expressed after normalization to the dose administered:

$$II' = \frac{\text{d.p.m./mg DNA}}{\text{d.p.m./kg body weight}}$$

This value was converted to the molar units,

$$II = \frac{\mu\text{mol thymidine incorporated/mol DNA nucleotide}}{\text{mmol thymidine applied/kg body weight}}$$

according to II = II' x 309 x $10^6$ on the basis of an average mol. wt. of 309 g/mol DNA nucleotides.

*Calculation of standard deviation.* The total variability (statistical counting error and fluctuations due to vial, scintillation cocktail, counter, external radiation and composition of the sample) for the counting of a DNA sample containing little radioactivity was assumed to be equal to the variability of DNA samples isolated from untreated animals held together with the treated ones. On the basis of 33 background values compiled for 12 months, a respective standard deviation of 1.89 c.p.m. (= 1 S.D.) was calculated. The standard deviation for a net radioactivity in a vial therefore was taken as: 1 S.D. = $\sqrt{(1.89^2 + (1.89/\sqrt{33})^2)} = 1.92$ c.p.m.

*Limit of detection for radioactivity in nucleoside analysis.* The total variability for each fraction of a h.p.l.c. analysis was calculated on a level of 1 standard deviation (1 S.D.) from 5 nucleoside analyses of control DNA digests. The mean background value for each fraction was calculated with an accuracy of 1 S.D./$\sqrt{5}$. The maximum possible difference between sample and background radioactivity was determined on an interval of ±2 S.D.

## Results

### *Comparison of isomers*

Table I compiles the radioactivities in mouse liver DNA isolated 10 h after oral administration of a high radioactivity dose of gamma- and alpha-[$^3$H]HCH. Under the assumption that the radioactivity is due to DNA-bound HCH molecules, the radioactivity can be expressed in the units of the CBI (14) after normalization to the dose administered. The last line of the Table I reveals the extremely low level of apparent DNA binding (CBI around 0.2) and shows that the alpha-isomer did not give rise to a higher CBI than the gamma-isomer

**Table I.** Specific activity of liver DNA of male NMRI mice, 10 h after oral administration of gamma- and alpha-[$^3$H]HCH.

| Isomer | gamma-HCH | | | alpha-HCH | | |
|---|---|---|---|---|---|---|
| Animal weight [g]; pool of 2 mice | 66.2 | 70.2 | 68.2 | 72.7 | 66.7 | 70.3 |
| Dose | | | | | | |
| [mg/kg] | 13.0 | 12.0 | 12.0 | 8.5 | 6.2 | 7.8 |
| [mCi/kg] | 44.9 | 41.5 | 41.5 | 27.5 | 21.2 | 26.8 |
| DNA Sp. act. | | | | | | |
| [d.p.m./mg] | 89.9 | 77.1 | 75.8 | 39.4 | 30.6 | 25.7 |
| ±1 S.D. | ±6.2 | ±7.3 | ±6.6 | ±4.8 | ±4.8 | ±4.1 |
| [CBI units] | 0.28 | 0.26 | 0.25 | 0.20 | 0.20 | 0.13 |
| ±1 S.D. | ±0.02 | ±0.03 | ±0.02 | ±0.02 | ±0.03 | ±0.02 |
| Mean ± SEM | 0.26 ± 0.02 | | | 0.18 ± 0.03 | | |

**Table II.** Specific activity of liver DNA of male NMRI mice, 10 h after oral administration of delta- and beta-[$^3$H]HCH.

| Isomer | delta-HCH | | beta-HCH | |
|---|---|---|---|---|
| Animal weight [g]; pool of 2 mice | 67.1 | 64.7 | 66.3 | 62.5 |
| Dose | | | | |
| [mg/kg] | 6.8 | 7.1 | 7.3 | 7.7 |
| [mCi/kg] | 23.5 | 24.5 | 25.2 | 26.7 |
| DNA Sp. act. | | | | |
| [d.p.m./mg] | 34.3 | 21.4 | <15.1 | <17.4 |
| ± 1 S.D. | ±12.6 | ±4.9 | | |
| [CBI units] | 0.20 | 0.12 | <0.08 | <0.09 |
| ±1 S.D. | ±0.07 | ±0.03 | | |
| Mean ± SEM | 0.16 ± 0.06 | | <0.08 | |

although the former is a markedly more potent carcinogen. This is the first indication to postulate that DNA binding cannot be the decisive activity for the tumor-inducing potential of the HCH's. This hypothesis is also supported by the finding that the delta-isomer which has never been found to induce tumors (16) also revealed a CBI of 0.16 (Table II). The beta-isomer did not give rise to detectable DNA radioactivity (Table II).

In order to check whether a difference between the gamma- and the alpha-isomer might be found at later times after the administration, the time dependence for the DNA binding was investigated. Figure 1 shows that the liver-DNA radioactivity reached the same plateau level of about CBI = 0.5 after 10 days. The time-dependent increase was faster in the first three days with the gamma-isomer, in accordance with the somewhat faster metabolism (17). It was also checked whether the absorption from the gastro-intestinal tract of the relatively high doses of chemical was different for the two isomers. This was not the case because it was found that the whole liver contained 2.1% and 2.2% of the radioactivity dose of the gamma- and the alpha-isomer, respectively, one day after the oral administration.

### *Comparison of mouse strains*

An additional hint for whether DNA binding could be the main mode of tumorigenic action of HCH should be obtained from studies with strains of mice that are of apparently different susceptibility to liver tumor induction by the gamma-isomer. The results given in Table III show that the three strains used all gave rise to similar CBI values although NMRI mice (CBI = 0.28) were found to be less susceptible to the tumorigenic action of gamma-HCl than B6C3F1 mice

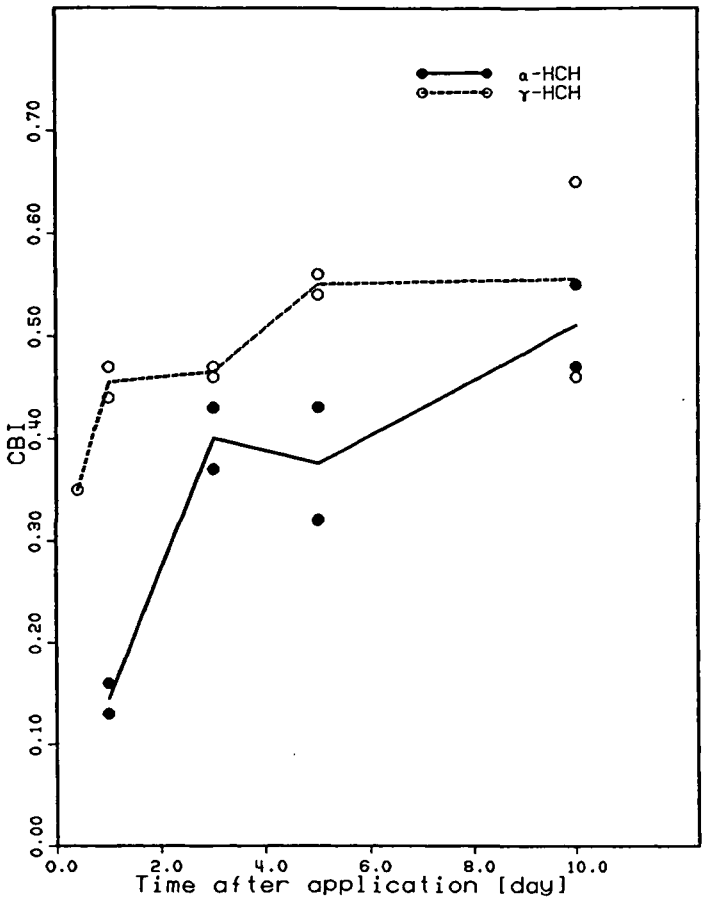


**Fig. 1.** Time dependence of the binding of alpha- and gamma-[$^3$H]HCH to mouse liver DNA. Groups of male NMRI mice were given p.o. ~30 mCi [$^3$H]HCH/kg and two animals were sacrificed each after 10 h (gamma-[$^3$H]HCH only), 1, 3, 5 and 10 days.

**Table III.** Specific activity of liver DNA of male NMRI, CF1, and B6C3F1 mice, 10 h after oral administration of gamma-[$^3$H]HCH.

| Strain | NMRI | | CF1 | | B6C3F1 | |
|---|---|---|---|---|---|---|
| Animal weight [g]; pool of 2 mice | 87.9 | 91.7 | 73.5 | 72.8 | 67.4 | 64.9 |
| Dose | | | | | | |
| [mg/kg] | 8.7 | 16.7 | 21.2 | 21.2 | 23.0 | 21.6 |
| [mCi/kg] | 30.0 | 57.7 | 73.0 | 73.0 | 79.3 | 74.3 |
| DNA Sp. act. | | | | | | |
| [d.p.m./mg] | 49.7 | 138.4 | 122.6 | 154.1 | 91.8 | 92.7 |
| ±1 S.D. | ±11.7 | ±11.6 | ±11.5 | ±8.6 | ±9.6 | ±12.8 |
| [CBI units] | 0.23 | 0.33 | 0.23 | 0.29 | 0.16 | 0.17 |
| ±1 S.D. | ±0.05 | ±0.03 | ±0.02 | ±0.02 | ±0.02 | ±0.02 |
| Mean ± SEM | 0.28 ± 0.04 | | 0.26 ± 0.02 | | 0.17 ± 0.02 | |

(CBI = 0.17) or CF1 mice (CBI = 0.26) (5–7).

*Contribution of contaminations to the DNA radioactivity*

It was assumed above that the DNA radioactivity was due entirely to DNA–HCH adducts. Other sources, such as biosynthetic incorporation of radiolabelled breakdown products of HCH entering the pool of DNA precursors, non-covalently bound HCH metabolites or protein contaminations might contribute all or a fraction of the radioactivity measured on the DNA. Any of these influences would make our argument for a non-mutagenic mode of action of HCH even stronger. A number of control experiments were performed to investigate some of the above-mentioned contributions.

**Table IV.** Specific activity of liver chromatin protein of male NMRI mice, 10 h after oral administration of [$^3$H]HCH.

| Isomer | gamma-HCH | | | alpha-HCH | | |
|---|---|---|---|---|---|---|
| Animal weight [g]; pool of 2 mice | 66.2 | 70.2 | 68.2 | 72.7 | 66.7 | 70.3 |
| Dose | | | | | | |
| [mg/kg | 13.0 | 12.0 | 12.0 | 8.5 | 6.2 | 7.8 |
| [mCi/kg] | 44.9 | 41.5 | 41.5 | 27.5 | 21.2 | 26.8 |
| Protein Sp. act. | | | | | | |
| [d.p.m./mg] | 6140 | 5170 | 3150 | 1374 | 1015 | 1126 |
| [PBI units] | 6.8 | 6.2 | 3.8 | 2.5 | 2.4 | 2.1 |
| Mean ± SEM | 5.6 ± 0.9 | | | 2.3 ± 0.1 | | |

*Contamination by protein-bound HCH.* The data given in Table IV show that chromatin protein was also radiolabelled, 10 h after [$^3$H]HCH administration. The specific activity was 42- to 68-fold and 33- to 44-fold higher in protein as compared with DNA for the gamma-, and the alpha-isomer, respectively. Protein contamination of DNA was shown to be lower than 0.2% as determined with radiolabelling of chromatin protein *in vivo* with [$^3$H]lysine or [$^{35}$S]methionine. Protein contaminations cannot therefore contribute substantially to the radioactivity measured on the DNA.

*Contamination of DNA by non-covalently bound HCH metabolites.* The control experiment where DNA was precipitated at different steps of the purification procedure showed that neither ether extraction nor dialysis resulted in a reduction of the specific [$^3$H]activity of DNA. This means that both lipophilic and hydrophilic metabolites had been removed completely from the DNA during the entire isolation procedure and that the DNA had been purified to constant specific activity.

*Nucleoside analysis*

Separation of the deoxynucleosides by h.p.l.c. after enzymatic digestion of liver DNA of alpha- and gamma-[$^3$H]-HCH treated NMRI mice (10-h-point) revealed that ~30–40% of the radioactivity eluted together with the optical density of the natural deoxynucleosides (Figure 2). This radioactivity is therefore most probably due to biosynthetic incorporation of breakdown products, for instance tritiated water. At a later elution time, a small (~10% of the radioactivity) but significant amount of radioactivity was detected. This is the region known to contain the more lipophilic nucleoside-carcinogen adducts. The formation of a nucleoside-HCH adduct therefore is highly probable. Up to 50% of the radioactivity of the DNA eluted in the first two fractions. This radioactivity can be due to DNA not completely degraded i.e., due to oligonucleotides which could still carry HCH adducts.

The same relative distribution of radioactivity among the three different elution regions was found with a duplicate DNA sample isolated 10 h after the administration of gamma-[$^3$H]-HCH and with a DNA pooled from 1-day, 3-day, and 5-day mice.

In order to further exclude the possibility that HCH metabolites non-covalently bound to DNA could be responsible for the presumed adduct peak in fraction 16 and 18 for the gamma- and the alpha-isomer, respectively, metabolites isolated from the liver of a gamma-[$^3$H]HCH-treated mouse

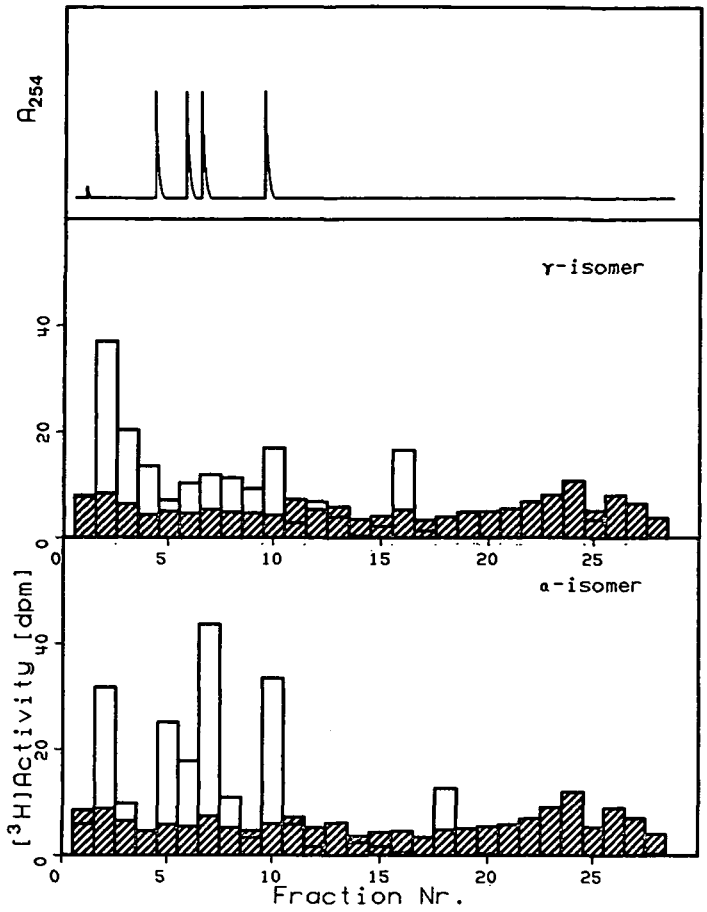


Fig. 2. Radioactivity profile of h.p.l.c. chromatograms of liver DNA of [3H]HCH-treated NMRI-mice, enzymatically digested to deoxynucleosides. The shaded area covers the range of the limit of detection for radioactivity in one fraction, calculated on an interval of ±2 S.D. Top, optical density profile, representing the natural deoxyribonucleosides in the order dC, dG, dT, dA; Center, DNA taken from a NMRI mouse, killed 10 h after oral administration of gamma-[3H]HCH; Bottom, DNA taken from an NMRI mouse, killed 10 h after oral administration of alpha-[3H]HCH. Similar profiles were obtained from a DNA pooled from 1-, 3-, and 5-day-mice (gamma-isomer).

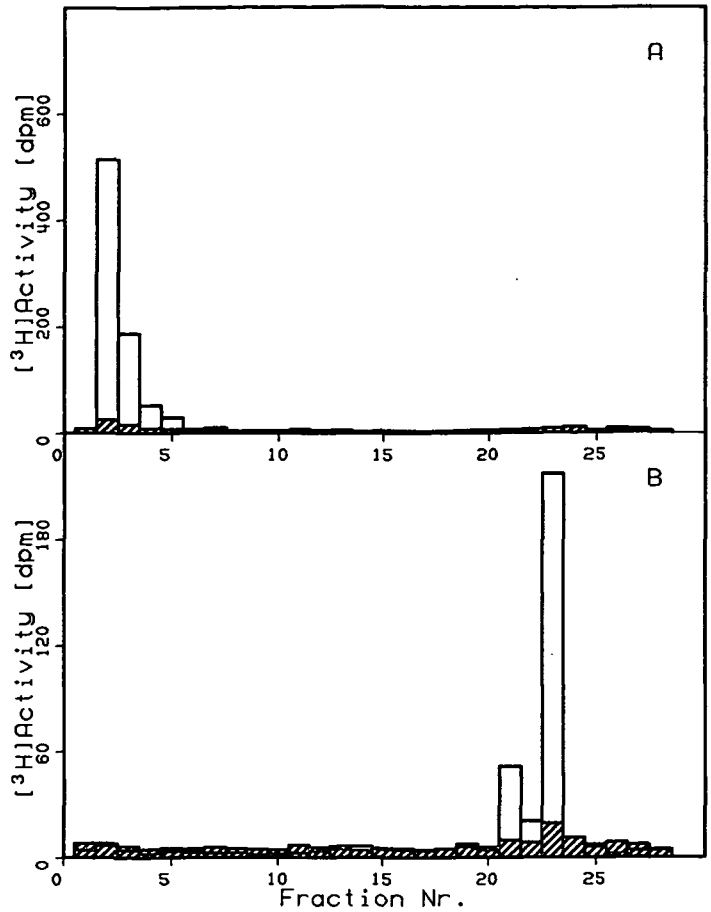


Fig. 3. H.p.l.c. analysis of metabolites of gamma-[3H]HCH isolated from mouse liver. The shaded area covers the range of the limit of detection for radioactivity in one fraction, calculated on an interval of ±2 S.D. A, hydrophilic fraction; B, lipophilic fraction.

were analysed on the same h.p.l.c. system as used for the nucleosides. Figure 3 shows that the hydrophilic metabolites eluted in the first three fractions. Since this is the region which also contained radioactivity in the h.p.l.c. analysis of the DNA nucleoside it cannot be excluded that such metabolites were closely but non-covalently associated with DNA. The association must have been so strong that the dialysis did not remove them and only upon enzymatic degradation of the DNA were they released.

Lipophilic metabolites eluted at a retention time of ~50 min. No radioactivity could be detected at retention times between 24 min and 48 min. These data indicate that the radioactivity peaks eluting after ~35 min represent HCH-deoxynucleoside-adducts and not metabolites set free during the hydrolysis of the DNA.

*True covalent DNA binding*

The control experiments described above have revealed that biosynthetic incorporation of radiolabel into DNA has taken place and that hydrophilic metabolites might have been strongly but non-covalently associated with DNA. For a calculation of a true CBI, these contributions have to be deducted from the values given in Tables I – III. A reduction by a factor of ~2 or 10 results, for the case where all early-eluting radioactivity is regarded as oligonucleotide-HCH adducts or for the case where only the nucleoside-HCH adduct peak is considered. On a most conservative approach, therefore, a CBI of <0.1 results.

*Protein binding*

In our experiments on DNA binding we also determined the level of non-extractable radioactivity in chromatin protein. This was done in a first place in order to determine whether contamination of DNA with protein of high specific radioactivity might simulate DNA binding. The protein-binding values are also low on an absolute level upon comparison with standard carcinogens (15), and, there was again no difference in protein binding between the two isomers (Table IV) or between the different strains of mice (Table V).

*Rate of DNA synthesis*

The animals received, 3.5 h before sacrifice, an i.p. injection of [14C]thymidine. The level of [14C]radioactivity on the DNA was then used as an index for DNA synthesis. The results summarized in Table VI show a tendency for the more potent alpha-isomer to induce a higher rate of DNA synthesis. It should be pointed out that these data were obtained from the identical animals used for the determination of DNA binding by [3H]HCH (Table I), where the alpha-isomer was even slightly less effective than the gamma-isomer.

**Table V.** Specific activity of liver chromatin protein of male NMRI, CF1 and B6C3F1 mice, 10 h after oral administration of gamma-[$^3$H]HCH.

| Strain | NMRI | | CF1 | | B6C3F1 | |
|---|---|---|---|---|---|---|
| Animal weight [g]; pool of 2 mice | 87.9 | 91.7 | 73.5 | 72.8 | 67.4 | 64.9 |
| Dose | | | | | | |
| [mg/kg] | 8.7 | 16.7 | 21.2 | 21.2 | 23.0 | 21.6 |
| [mCi/kg] | 30.0 | 57.7 | 73.0 | 73.0 | 79.3 | 74.3 |
| Protein Sp. act. | | | | | | |
| [d.p.m./mg] | 3300 | 7610 | 7910 | 9360 | 4560 | 4330 |
| [PBI units] | 5.5 | 6.5 | 5.4 | 6.3 | 2.9 | 2.9 |
| Mean ± SEM | 6.0 ± 0.5 | | 5.8 ± 0.5 | | 2.9 ± 0.02 | |

**Table VI.** Incorporation of [$^{14}$C]thymidine into liver DNA of male NMRI mice, 3.5 h after i.p. injection, and 10 h after oral application of [$^3$H]HCH.

| Isomer | gamma-HCH | | | alpha-HCH | | |
|---|---|---|---|---|---|---|
| Animal weight [g]; pool of 2 mice | 66.2 | 70.2 | 68.2 | 72.7 | 66.7 | 70.3 |
| Dose [$^{14}$C]TdR | | | | | | |
| [μg/kg] | 40.0 | 30.5 | 40.0 | 36.8 | 35.5 | 28.8 |
| [μCi/kg] | 10.0 | 7.6 | 10.0 | 9.2 | 8.9 | 7.2 |
| DNA Sp. act. | | | | | | |
| [d.p.m./mg] | 184 | 149 | 181 | 284 | 180 | 180 |
| Incorp. Index | 2560 | 2710 | 2520 | 4280 | 2820 | 3460 |
| Mean ± SEM | 2600 ± 60 | | | 3500 ± 400 | | |

## Discussion

The previous section has provided good qualitative evidence for a DNA–HCH adduct. A comparison among strains and isomers makes it highly unlikely, however, that this type of genotoxic activity is the decisive mode of tumorigenic action. In addition, a quantitative analysis of the level of DNA binding favours a non-genotoxic mode of tumorigenic action. A quantitative correlation of CBI *versus* carcinogenic potency expressed in $TD_{50}$ units (i.e., the daily dose estimated to induce a tumor in 50% of the animals treated for life), has shown that CBI of the order of $10^3 - 10^4$ are found with strong genotoxic carcinogens, of 100 for moderate carcinogens and of 1–10 for weak carcinogens (18). Since alpha-HCH has to be classified as a moderate tumor-inducing agent with an approximate $TD_{50}$ value of 0.1 mmol/kg/day for mouse liver, a CBI for liver DNA of $\sim 10^2$ would be required if its mode of action was by DNA binding. The measured value of $<0.1$ is one thousand times lower.

Among the many possible mechanisms of tumorigenic activity not related to DNA binding two aspects were amenable to an assay within the present experimental set-up. Firstly, binding to protein and the concomitant cytotoxicity might be envisaged. Our results showed, however, that protein binding cannot be an important contribution to the proposed non-mutagenic mode of tumor induction.

Another possibility for a non-mutagenic mode of action, the stimulation of cell division, was also tested simultaneously with the determination of DNA binding. These results gave some indication for a higher activity of the alpha-isomer although the experimental set-up was not ideal for the determination of this activity. It has been shown in rats that the induction of DNA synthesis after administration of various tumor promoters is largest after a time period of ~20 h (19,20). In our experiments, we used mice and the interval was only 6.5 h. Therefore, the borderline effect observed with the alpha-isomer might have become more pronounced if a time dependence had been investigated. Additional evidence along these lines is available from two-stage long-term carcinogenicity studies with rats where it has been shown that alpha-HCH accelerated the manifestation of malignant liver tumors after initiation of the carcinogenic process by a single dose of diethylnitrosamine (21).

Chemical carcinogens are normally divided into two large classes of mutagenic and non-mutagenic carcinogens (22). As suggested by Radman and Kinsella (23) there may not be a clear separation of these two groups of activities and it must be expected that there are carcinogens acting on more than only one level.

With HCH, we are faced with a situation where a minute DNA binding was shown for the alpha- and the gamma-isomer. Much additional information was given, however, to show that this activity cannot be responsible alone for the tumors induced and some hypotheses were presented and in part supported by experimental data. For a risk evaluation in man, a mutagenic risk by DNA binding after exposure to mg amounts of lindane seems negligible. Although species differences with respect to the formation of reactive metabolites cannot be excluded we do have evidence that binding of gamma-HCH to liver DNA in the rat is similar to the data obtained in mice so that there are at least no indications for large species differences with respect to metabolism. For the more important events not related to DNA binding, an extrapolation of animal data to man must be based upon the elucidation of the specific mechanism of tumor induction by HCH in the animal, and a test of whether similar activities are found in man at dose levels that are by orders of magnitude lower than those used in the long-term bioassays.

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